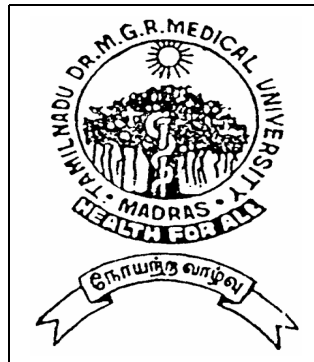


A STUDY ON THE OCCURRENCE AND SUBTYPING OF ACUTE LEUKEMIAS BASED ON MORPHOLOGY AND CYTOCHEMISTRY

DISSERTATION SUBMITTED FOR

M.D. (PATHOLOGY)

MARCH 2008



THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY

CHENNAI – TAMILNADU

CERTIFICATE

This is to certify that the dissertation entitled “**A STUDY ON THE OCCURRENCE AND SUBTYPING OF ACUTE LEUKEMIAS BASED ON MORPHOLOGY AND CYTOCHEMISTRY**” submitted by **Dr. M.SINTHA** to the Faculty of Pathology, The Tamilnadu Dr. M.G.R. Medical university, Chennai in partial fulfillment of the requirement for the award of M.D. Degree in Pathology is a bonafide work carried out by her during the period June 2005 – Nov 2007 under my direct supervision and guidance.

Dr. D. Gomathinayagam, M.D.,
Professor and Head,
Department of Pathology,
Madurai Medical College,
Madurai.

ACKNOWLEDGEMENT

“No academic endeavour is single handedly accomplished this work in no exception –
Anonymous.

I am extremely grateful to my respected Professor and Guide **Dr. D.Gomathinayagam, M.D.**, Professor and Head of Department of Pathology for his valuable guidance at every stage, constant encouragement and advice which have been the motivating forces in bringing forth this piece of work.

My sincere thanks to **Dr. Mrs. Usha Ravikumar M.D.**, Additional Professor Pathology for her valuable suggestions and encouragement during this period.

I owe my gratitude to all Assistant Professors for their valuable suggestions and guidance at every stage in this study.

I am indebted to all the technical staff of the Department for their immense help in carrying out this study.

I am grateful to the Dean, Madurai Medical College and Government Rajaji Hospital, Madurai for permitting me to carryout this study.

I am grateful to my family members and friends for the enduring patience and support during the study period.

Last but not least my sincere thanks to Mr.S.Ganesh Babu Medianett for the computerized colorful presentation of the data.

CONTENTS

Chapter	Title	Page No.
1.	INTRODUCTION	1
2.	AIM OF STUDY	4
3.	REVIEW OF LITERATURE	5
4	MATERIAL AND METHODS	42
5	OBSERVATION AND RESULTS	44
6	DISCUSSION	53
7	SUMMARY	62
8	CONCLUSION	63
9	BIBLIOGRAPHY	
	ANNEXURE – I (PROFORMA)	
	ANNEXURE – II (STAINING TECHNIQUES)	
	ANNEXURE – III (MASTER CHART)	

INTRODUCTION

Leukemia is the term used for haematopoietic neoplasm, presenting with wide spread involvement of bone marrow and peripheral blood by blasts and is the most common cancerous disorder in children¹.

Virchow recognized leukemia as a distinct disease in 1845 when he used the term weisses blut to describe the findings in an autopsy in which the ratio of red corpuscles to “colorless (in mass white)” was reversed². He used the term leukemia for the first time in 1847 and subsequently, described lymphatic and splenic forms³.

Friedreich recognized acute and chronic types in 1857.

Neumann, in 1868, identified the bone marrow as the origin of leukemia and used myelogenous as a provisional term that has been validated over time.

The first case of Acute Myeloid Leukemia (AML) was originally termed acute non lymphocytic leukemia in 1900.

During the first half of the century most of the subcategories of myeloid leukemia were identified by light microscopy with the aid of cytohistochemical stains and were described based on the resemblance to normal hematopoietic precursors. The recognition of the Philadelphia chromosome in chronic myeloid leukemia (CML) by Norwell and Hungerford in 1960 was the first demonstration of recurring chromosomal abnormality in leukemia.

In 1976 FAB classification was proposed, with several subsequent modifications. The FAB group defines seven subsets of AML. Four based on the percentage of maturing cells and three based on lineage. FAB classifies acute lymphoblastic leukemia (ALL) into three types based on morphology as L1, L2, and L3.

In the latter half of the last century, numerous cytogenetic abnormalities, including specific translocations, were identified in subtypes of AML and led to changes in classifications, as proposed in 1997 by the WHO⁴.

Cytogenetics has profound effect on prognosis and treatment.

The WHO subdivides AML into true denovo AML occurring predominantly in young to middle-aged adults, with recurring cytogenetic translocations or inversions and MDS related AML occurring in elderly adults often with complex chromosomal abnormalities. The WHO recognizes therapy related AML and retains the morphologic subcategories of the FAB in cases not otherwise classified. The category of acute leukemia of ambiguous lineage is also added.

As per WHO classification (2001) the ALLs are separated under three broader categories, precursor T-cell, precursor B-cell, and mature B-cell neoplasm based on their Immunophenotype⁵.

The high cost of cytogenetics and Immunophenotypic studies stands in way of routine application in all patients.

In spite of advances made in classification and prognostic factor delineation, it is essential to classify leukemias basically into AML and ALL as they differ significantly in management and prognosis. This can very well be done by combination of morphology and cytochemistry which has a sensitivity of more than 92%⁶. In a developing country like India all the patients do not have accessibility to cytogenetics and Immunophenotypic studies. Hence classification was hitherto done by morphology alone in the past decades. This situation necessitated the introduction of cytochemistry, which is an improvement over the previous method, though not up to the level of precision obtained in cytogenetic studies. Hence the present study has been taken up to subtype leukemias using morphology and cytochemistry.

AIM OF THE STUDY

- To evaluate the clinical and haematological profile of patients with leukemia admitted to Government Rajaji Hospital, Madurai.
- To classify leukemia based on morphology.
- To employ cytochemistry for precise subtyping.
- To evaluate the correlation between morphology and cytochemistry.
- To employ immunophenotyping in selected cases.

REVIEW OF LITERATURE

Blood contains several types of cell each having a distinct appearance and function. The process of production of various cells from the haematopoietic stem cell is known as haematopoiesis. During the early embryonic period haematopoiesis occur in the embryonic yolk sac, then during development it shifts to the liver, spleen and then ultimately to the bone marrow which becomes the important site of blood cell production.

NORMAL HAEMATOPOIESIS

THE ERYTHROID SERIES

The pro-erythroblast is the least mature of the erythroid series; it undergoes rapid division and gives rise to basophilic erythroblasts. Next stage is the polychromatic erythroblast; the characteristic polychromatic appearance of the cytoplasm is derived from the mixture of the basophilic ribonucleic acid (RNA) and acidophilic hemoglobin. Proliferative activity ceases after this stage. Next stage in development is the orthochromatic erythroblast which has a small pyknotic nucleus. It develops into the mature erythrocyte.

THE GRANULOCYTIC SERIES

The myeloblast is the morphologically identifiable earliest precursor of myeloid series. There are no typical granules in the moderately basophilic cytoplasm. Nuclear chromatin is fine with typically prominent two to three nucleoli, there may be up to six nucleoli. It develops into a promyelocyte, it has some cytoplasmic granules and a slightly

coarser appearing chromatin, nucleoli are still present. The myelocyte is the next stage. Granulocyte precursors undergo active proliferation upto the myelocyte stage.

Subsequent steps in the maturation process, involve progressive changes in the conformation of the nucleus from round in the myelocyte through Metamyelocyte, stab and the segmented in the mature form.

THE LYMPHOID SERIES

Lymphoblasts are slightly smaller, the ratio of the nucleus to that of the cell is greater, and the number of nucleoli per nucleus tends to be fewer. Differentiation into mature forms does not proceed along such morphologically well-demarcated steps as with the other blood cell series, and the morphological features largely reflect whether the cell is engaged in proliferative activity or is in the dormant state.

ACUTE LEUKEMIA DEFINITION

Acute leukemias are the result of accumulation of early myeloid or lymphoid precursors in the bone marrow, blood and other tissues and are thought to arise by somatic mutation of a single cell within a minor population of stem or early progenitor cells in the bone marrow or thymus. As per WHO classification a blast count of above 20% is sufficient for the diagnosis of acute leukemia as against 30% blasts prescribed earlier⁷.

EPIDEMIOLOGY

The overall age adjusted incidence of leukemia varies from 0.34% to 1%⁸.

ALL is the most common paediatric malignancy accounting for three-fourth of all newly diagnosed leukemias and one-fourth of all cancers in childhood. It constitutes about 62.5 to

75% of cases in children⁹.

The most common age group at presentations for ALL is 2 to 6 years¹⁰.

In contrast to ALL, the incidence of AML is quite constant from birth throughout the first 10 years. Following a slight peak in adolescence the incidence remains nearly constant upto 55 years of age, after which it increases progressively. The case ratio of ALL to AML in children under 15 years is 4:1 approximately the reverse of the ratio in adults¹¹.

In children ALL, L1 constituted about 63.3%, L2- 36.3%¹¹. While other reviews quote L1 being about 80 to 85%, L 2 -15% and L3 –1.2%¹². ALL L2 blasts were more common in adults and L1 blasts were more common in children. In adults L1 forms 21% and L2 68% of cases¹³.

Among AML, M2 is the most common (27 – 29%) followed by M4 (16 – 25%) and then M5 (13 – 22%)¹⁴.

Male preponderance is a salient feature of childhood tumors¹¹.

In infants AML M4, M5 is more common¹⁵. Where as other reviews quote ALL to be common¹⁶.

ETIOLOGY

The causes for ALL in adults are largely unknown. Atomic nuclear fallout¹⁷ and high level of benzene exposure are accepted as causes for ALL¹⁸.

As far as AML is concerned environmental, occupational and genetic factors play a role in pathogenesis.

Individuals with genetic disease that promote genomic instability such as Bloom's syndrome, Fanconi anemia, and Ataxia Telangiectasia are at increased risk for development of

acute leukemia¹⁹. In addition, both Down's syndrome and Neurofibromatosis Type I²⁰ are associated with an increased incidence of childhood leukemias.

The ratio of ALL to AML in children with Down's syndrome follows the usual childhood distribution except during the first three years of life when AML (FAB M 7) is more likely to occur than ALL²¹.

Infantile monosomy 7 syndrome can evolve into aplastic anaemia.

Fanconi anaemia confers the highest risk for developing AML.

Incidence of leukemia has been increasingly reported in workers involved in shoe making, organic synthesis and rubber and paint manufacturing.

Ionizing radiation is also implicated in the etiology of leukemia. An emerging set of observations indicates that intensive chemotherapy can induce second lymphoid or myeloid malignancies²².

Drugs have also been linked to AML, with the most convincing evidence pertaining to the antineoplastic agents, particularly alkylating agents and topoisomerase II inhibitors. The time interval between onset of leukemia and exposure to alkylating agent ranges from 1 to 28 years and is most commonly in the range of 5 to 9 years²³.

PATHOBIOLOGY

Leukemia is associated with changes in chromosome number, translocation and inversions of genetic material. These alterations result in activation or increased transcription of oncogenes.

In case of lymphoid neoplasms many of the oncogenic rearrangements stem from mistakes during events that occur during antigen receptor gene expression. B and T cell

progenitors express a V (D) J recombinase activity that cuts DNA at specific sequences within the immunoglobulin and T cell receptor loci and many pathogenic rearrangements seen in lymphoid neoplasm are caused by inappropriate joining of these sites to sequences flanking proto oncogenes.

The most common cytogenetic abnormality in ALL in adults is the abl bcr fusion gene. Other gene rearrangements result in loss or gain of function mutations involving transcription factors that are involved in normal haematopoiesis.

In AML loss or inactivation of tumor suppressor gene is implicated which have key regulatory function in controlling cell cycle progression. There is evidence of two hit model of leukemogenesis. Fusion genes (AML 1/ETO, CBF/SMMHC) and PML/RAR ALPHA (promyelocytic leukemia/ retinoic receptor) involved in specific chromosomal abnormalities. t (8;21), inv 16 and t (15;17) impair differentiation and apoptosis, whereas other classes of genetic changes such as mutations in FLT 3 and NRAS promotes proliferation. Topoisomerase II inhibitor administration is associated with AML with 11q 23 and 21q 22 translocation.

SOME KEY CLINICAL FEATURES

AML and ALL differ in clinical presentation response to the chemotherapy and course.

Fatigue and lethargy correlate with the degree of anemia,

DIC is commonly associated with APL.

Petechia and ecchymoses are the most common manifestations of hemorrhage at the time of diagnosis in 40% to 60% of patients with acute leukemia^{24,25}.

In a large series of patients with acute leukemia, leukemia cutis occurred in 11% of the patients with AML and in 1.3% of patients with ALL, and retinal bleeding occurs in

approximately 15 % of patients in acute leukemia at diagnosis.

Skin involvement is rare in ALL. When it occurs it is associated with pre B cell phenotype²⁶.

Clinical features of AML M5 are cutaneous lesions, gum infiltration, and CNS disease. CNS involvement has been reported in 3 to 22% of patients and is more common in acute monocytic leukemia than in other subtypes of AML²⁷.

CNS involvement by leukemia occurs in 2% of patients and is manifested by headaches vomiting and cranial nerve palsy²⁸. Ferry JA et al had noted that testicular involvement is less common in AML than in ALL²⁹.

One third of patients with ALL have bleeding symptoms at diagnosis which is less frequent than in patients presenting with AML.

Generalised lymphadenopathy occurs in 50% of ALL cases and in insignificant number of AML cases³⁰.

Gum hypertrophy is present in 3-5% of acute leukemia cases of which 88.9% are AML³¹.

Myeloid sarcoma or Myeloblastoma is an extra medullary tumor that occurs in 2-14% of cases of AML, common sites being the orbit and para nasal sinuses

LAB INVESTIGATIONS

Patients should have a battery of diagnostic laboratory test to confirm the diagnosis, subcategorize for prognostic classification and plan for appropriate therapy. These include complete blood count with examination of peripheral blood smear, electrolyte measurements, creatinine, hepatic enzymes, uric acid, calcium, albumin levels, chest radiography, CT, lumbar

puncture, bone marrow aspiration and biopsy.

There is elevation of serum uric acid levels although gout is a rare complication. Hyperphosphatemia and hypocalcemia can occur.

Serum lactate dehydrogenase is elevated.

Coagulation parameters are normal and disseminated intra vascular coagulation is rare.

The Revised criteria for CNS involvement at diagnosis, As quoted by Mastrangelo R³² is as follows -

CNS-1 - one blast cell

CNS-2 - <5 WBC/micro litre with blasts cells

CNS-3 -> 5 WBC/micro litre with blasts or Cranial nerve involvement.

PERIPHERAL SMEAR AND BONE MARROW

Despite the advent of modern ancillary techniques, morphologic examination and cytochemical staining of well-prepared air-dried peripheral blood smears and bone marrow smears are critical in the pathologic diagnosis and classification of acute leukemias. Anaemia is more common in AML and is predominantly normocytic and normochromic. Nucleated red cells are more common in AML. Alternatively ALL may present with leukoerythroblastic picture. Adult patients with ALL have normal or only moderately elevated WBC count at diagnosis. 15 % of patients present with marked hyper leukocytosis. One third of patients present with platelet count less than $25000 \times 10^6/\text{litre}^{33}$.

Subleukemic presentation is seen in 21.90% of cases³⁴.

MORPHOLOGICAL FEATURES OF LYMPHOBLAST

Cytologic features	L1	L2	L3
Cell size	Small cells predominate	Large, heterogeneous in size	Large and homogeneous
Amount of Cytoplasm	Scant	Variable, often mod. Abundant	Moderately abundant
Nucleoli	Not visible, or small and inconspicuous	One or more present, often large	One or more present often prominent
Nuclear Chromatin	Homogeneous in any one case	Variable, heterogeneous in any one case	Finely stippled and homogeneous
Nuclear Shape	Regular, occasional clefting or indentation	Irregular, clefting and indentation	Regular oval to round
Basophilia of cytoplasm	Variable	Variable	Intensely basophilic
Cytoplasmic vacuolation	Variable	Variable	Prominent

SCORING SYSTEM TO DISTINGUISH L1 from L2

Features	Description	Scoring
Nuclear/Cytoplasmic ratio	>20% of cell area is cytoplasm in >25% of cell	-1 (favors L2)
	<20% of cell area is cytoplasm in >75% of cell	+1 (favors L1)
Nucleoli	One or more prominent nucleoli in <25% of cell	-1
Nuclear membrane	Absent or inconspicuous in >75% of cell	+1
	Irregular in >25% of cells	-1
Cell Size	>50% of large cells	-2
	L>2xdiameter of lymphocytes.	

Occasionally the morphologic distinction between L1/L2 and L3 may be obscured because L1/L2 blasts may sometimes show significant cytoplasm vacuoles.

Granular variant of ALL shows presence of granules in some of the blasts; these are negative for MPO and faintly positive for PAS and Sudan black. Another study found no prognostic difference for this granular variant of ALL . The granules are larger and less abundant than in AML.

Azurophilic granules are observed in 10% of L1 and L2 blasts, they may be confused with myeloblasts and reliable differentiation requires cytochemical stains and

immunophenotyping

Myeloblasts are grouped into 3 types of blast cells. All have central nuclei with fine uncondensed chromatin and prominent nucleoli. Type I blasts do not have cytoplasmic granules but type II blasts have small number of primary granules. Type III blasts are similar to Type II blasts with the exception of more abundant azurophilic granules.

CYTOCHEMISTRY

Cytochemistry is the study of chemical elements found in cells. These elements may be enzymatic such as peroxidases or nonenzymatic such as lipids and glycogen.

The principal uses of cytochemistry are

- To characterize blast cells in acute leukemia as myeloid.
- To identify granulocytic and monocytic components of AML.
- To identify unusual lineages occasionally involved in clonal myeloid disorders (basophils and mast cells).
- To detect cytoplasmic abnormalities and enzyme deficiencies in myeloid disorders such as MPO deficient neutrophils in MDS and acute leukemia.

As per Hayhoe, cytochemistry is particularly useful for the characterization of immature cells, when they are performed on peripheral blood films, bone marrow aspirates and touch preparations from lymph node³⁵.

Cytochemical results were confirmed by ultrastructural studies in 90% of the cases. Clinically significant discrepancies between cytochemical and ultrastructural interpretations were rarely found³⁶.

Elghetany MT, et al in their study had found that, some cases of M1, M5a, M7, and L2

were morphologically similar³⁷. In such cases, cytochemical stains can provide an inexpensive and readily available diagnostic tool

Gassmann in 1997 evaluated the morphological findings in 150 consecutive cases of T-lineage acute lymphocytic leukaemia (T-ALL). Cytochemistry including PAS staining and acid phosphatase reaction were proved to be of limited value for the diagnosis of ALL³⁸.

Sharma JS and Mohindroo S had found that the concordance with morphology alone was 75% which improved to 92% when cytochemistry is included⁶.

The cytochemical profile of blasts is in concordance with immunophenotype, particularly in more differentiated AML subtypes, M3, M4 and M5³⁹.

The stains commonly used are

- 1) Myeloperoxidase (MPO)
- 2) Sudan Black B (SBB)
- 3) Non specific esterase (NSE)
 - Alpha naphthyl acetate esterase
 - Alpha naphthyl butyrate esterase
- 4) Specific esterase
- 5) PAS

MYELOPEROXIDASE

Bainton D had found that Myeloperoxidase (MPO) is located in the primary and secondary granules of granulocytes and their precursors, and in specific granules of eosinophil⁴⁰. It is also present in basophil granules. Reaction is weakly positive in monocytes.

MPO splits hydrogen peroxide, and in the presence of a chromogenic electron donor forms an insoluble reaction product. Various benzidine substitutes are used of which 3, 3'-

Diaminobenzidine (DAB) is the preferred chromogen. The reaction product is somewhat stable, insoluble and nondiffusible⁴¹.

Alternate nonbenzidine based techniques employ 4-chloro-1-naphthol. It gives very crisp staining, but is soluble in some mounting media and immersion oil⁴².

3-amino-9-ethylcarbazole is also employed; it shows some diffusibility and does not stain as strongly as DAB⁴³.

Cheson BD et al, had concluded that Lymphocytes and erythroid precursors are negative and demonstration of MPO activity in leukemic blasts establishes a diagnosis of AML and added that in cases of ALL residual normal MPO-positive immature precursors are present in small numbers⁴⁴.

Glick AD et al reported MPO positivity in 19 of 83 B cell ALL, 16 of these cases had MPO positive blasts of more than 10%⁴⁵. McKenna reported MPO positivity in 20 out of 150 cases of T cell ALL. Most of the cases showed weak positivity in the range of 5% but one case showed 20% MPO positivity⁴⁶. The sensitivity and specificity of MPO stain is 69% and 100% respectively⁴⁷.

At the Anderson cancer center, cases of ALL with 20% to 60% MPO positivity in the blast population with absence of myeloid markers are reported. All cases were typical pre-B cell ALL with expression of CD19, CD10, CD34, and TdT.

SUDAN BLACK B

Sheehan H and Storey G proposed an improved method of staining neutrophil granules

with Sudan black B stain, they proposed that SBB stain the granule membranes, the pattern closely resembling that of MPO but SBB positivity is stronger than MPO⁴⁸.

Lillie R and Burtner H had proposed that the sudanophilia of neutrophil granules is stable in relation to peroxidases. The reaction product is black and granular⁴⁹.

The SBB is non specific in diagnosing AML, as some ALL (especially the granular ALL) may have a weak SBB positivity. Lymphoblasts show a nongranular smudgy positivity⁵⁰.

Monocytes may contain sudanophilic granules and monocytic leukemia may be SBB positive, Erythroid precursors and lymphoblasts are negative.

PAS

PAS is present in many normal blood cells. The reaction product is red, with intensity ranging from pink to bright red. Cytoplasmic positivity may be diffuse or granular⁵¹.

Most cases of ALL have a characteristic “block” PAS staining pattern. This Staining pattern distinguishes ALL blasts from other cell types (such as granulocytes, megakaryocytes, and monocytes) that can have diffuse cytoplasmic PAS staining.

The finding of block PAS positivity is not specific for ALL, and block PAS reactivity can be seen in some acute myelogenous leukemia such as acute erythroblastic leukemia⁵².

As per J S Lilleyman et al, the PAS reaction features in ALL is strongly associated with a poor prognosis.

PAS staining do not distinguish myeloid, T-, B-, or non-T/non-B cell lines. Characteristic reaction (large number of coarse granules against a clear background forming a ring around the nucleus) is found in erythroblastic leukaemia cell line and along myeloid series its intensity increased in more mature cells.

Snower DP et al, in their study of 51 cases found that the sensitivity and specificity of

the PAS stain alone for lymphoblastic leukemia was 52% (15 true positives of 29) and 81% (four false positives), respectively⁵³. The sensitivity of a cytochemical-staining combination of PAS positivity and myeloperoxidase, Sudan black B, and alpha-naphthyl butyrate esterase negativity in defining cases of lymphoblastic leukemia remained at 52%; however, the specificity of this combination for lymphoblastic leukemia was 100% (no false positives). Thus, a positive PAS stain, in combination with negative myeloperoxidase, Sudan black B, and alpha-naphthyl butyrate esterase stains, continues to have a diagnostic role in the distinction between lymphoblastic and myeloblastic leukemia, and greater immunologic sophistication serves to support this position.

ESTERASES

Leukocyte esterases are a group of enzymes that hydrolyse acyl or chloroacyl esters of alpha naphthol AS.

9 esterase isoenzymes are present which fall into 2 groups in gels. Bands 1, 2, 7, 8 and 9 correspond to specific esterase of granulocytes, they stain specifically with Naphthol AS-D Chloroacetate esterase. Bands 3, 4, 5 and 6 correspond to non-specific esterase, they stain with Alpha –Naphthyl acetate esterase and Alpha –Naphthyl butyrate esterase.

Moloney W had demonstrated esterase activity in monocytes using naphthol ASD chloroacetate⁵⁴.

Yam L et al had described that the most commonly used substrates to demonstrate NSE activity are naphthyl butyrate and alpha naphthyl acetate, the reactivity of which in monocytes is granular or diffuse depending on the color developer used⁵⁵.

Li C et al had described that, monocytic NSE is inhibited by Na F, In

megakaryoblasts, the NSE activity is partially resistant to NaF inhibition, megakaryoblasts are negative with ANB staining. Lymphoblast may be positive for NSE, with variable inhibition by NaF⁵⁶.

Nonspecific esterase, and chloroacetate esterase reaction are found in myelocytic and monocytic cell lines with the reaction intensity increasing progressively in more mature cells.

NSE stains are useful in differentiating M2 from M4.

ACID PHOSPHATASE

Cytochemically demonstrable Acid phosphatase (AP) is ubiquitous in haematopoietic cells. Focal acid phosphatase reaction is found in all T-ALL cell lines, whereas myeloid/monocytoid lines have semicircular distribution and B-cell lines have cytoplasmic distribution of activity. Acid phosphatase activity appeared to decline with maturation along both myeloid and T-cell lineage. Its main diagnostic use is in the diagnosis of T cell acute leukemia and Hairy cell leukemia⁵⁷. But these are more reliably diagnosed and characterized by immunophenotyping when this is available.

IMMUNOPHENOTYPING

The development of monoclonal antibody against cell surface markers of blood cells and their conjugation with certain fluorochemicals markedly contributed to the application of flow cytometry in the study of normal haematopoiesis.

Commitment to B cell differentiation is indicated by the appearance of CD19 & CD10. Typical phenotype of peripheral B lymphocyte is CD19+, CD20+, CD21+ and CD22+. Majority of blood T lymphocytes are CD2+, CD3+ and CD7+ and express either CD4

or CD8.

CD33 is the earliest marker for myeloid differentiation. Immature myeloid cells become CD13+ followed by appearance of CD15 and CD11b. in contrast monocytes are strongly CD33+ and weakly CD15 and CD4 positive.

IMMUNOPHENOTYPING IN HEMATOLOGICAL MALIGNANCY

Markers specific for hematological malignancy exist only when a new protein is encountered encoded by a gene resulting from fusion of two normal genes. E.g. (bcr-abl). Diagnosis by immunophenotyping can be still accurately made by routine leukocyte antigen and other proteins which are expressed on cell surface. It is based on

- 1) Presence of marker in amount significantly more than normal.
- 2) Aberrant phenotype resulting
 - a) Expression of marker from lineage on which it is not normally expressed.
 - b) Asynchronous antigen expression.

The immunologic relevance of the immunophenotype to the FAB subtypes M1, M2, M4, and M5 was possible in greater than 80% of cases. Immunophenotyping is to be done at least in cases with negative or inconclusive cytochemistry and has suggested that the best method for typing acute leukemia is by a study using a combination of morphology, cytochemistry and immunophenotyping.

CD10 expression is lower in infants and in children.

In the study on lineage promiscuity in leukemia, CD7 which is the most sensitive marker of T cell ALL is found in 20% of AML cases.

Bucheri V etal had proposed a scoring system for a diagnosis of biphenotypic

leukemia⁵⁸.

Points	B cell	T cell	Myeloid
2	CD79a ,CD 22 cytoplasmic IgM	CD3	MPO
1	CD 19 ,CD10	CD2 CD5	CD33 CD13
0.5	TdT	TdT ,CD7	CD14 ,CD15, CD11c,CD11b

Biphenotypic acute leukemia is established when score from two separate lineages is more than 2. Recent studies include CD117 as a highly specific myeloid marker equivalent to 2 points and T cell receptor a highly specific T cell marker with 2 points.

Expressions of myeloid antigen marker are slightly more frequent in B-lineage ALL than in T-lineage ALL but have no prognostic value⁵⁹.

For therapeutic purposes one need only to distinguish T cell & mature B cell cases from those originating from precursor B cell.

It is concluded that morphology, histochemistry and immunological phenotype on bone marrow smears are the main diagnostic basis for AML-M 0. The use of multiple monoclonal antibodies for staining may improve the accuracy as CD3/CD13 were positive, and CD2/CD3/CD10/CD19/CD22 were negative in AML M0.

A small subset of mature B cell ALL with surface immunoglobulin expression and chromosomal translocation t (8;14) are shown to express TdT.

Mature B-cell ALL is characterized by expression of surface immunoglobulin, either kappa or lambda, in addition to other markers common to B-lineage ALL, including CD10, CD19, CD20, and CD22.

Bucheri V. et al had observed that precursor B cells typically have TdT reactivity and coexpression of CD10, CD19 and absence of surface immunoglobulin expression. They also observed that some cases with earliest precursor immunophenotype may express TdT and HLA DR only with no T cell or myeloid associated markers these are termed as unclassified or undifferentiated ALL.

They concluded that most cases of ALL expressing myeloid markers not fulfilling the diagnostic requirements of biphenotypic leukemia have been found to have prognostic features similar to those that do not express myeloid antigens. Mixed lineage acute leukemias are cases which have two different population of cells, as MPO positive blasts in an otherwise morphologically and immunophenotypically typical ALL.

T cell ALL are subclassified into different stages corresponding to normal thymocyte development , the early subtype is negative for surface CD3 and is either double positive or double negative for CD4 and CD8 , the later subtype is surface CD3+and positive for CD4 or CD8 and not both. Recent studies do not find any prognostic differences in this sub classification of T cell ALL.

It has been concluded that evaluation of the cytochemical profile in connection with immunophenotype helps to classify the AML patients to relevant subtypes with more accuracy.

Myeloid associated antigens are identified in 45.6% of pre B cell ALL and in 32% of T cell ALL. , CD13 and CD33 are commonly associated myeloid antigens; these are designated as My + ALL.

Elghetany MT et al had concluded that all cases of acute leukemia are easily subtyped by morphology and cytochemistry. Immunophenotyping, karyotyping, and molecular analysis of DNA and RNA of leukemia cells may be required to define cell lineage. Lineage assignment is

based on the

- 1) morphological features
- 2) cytochemistry and
- 3) immunophenotyping

CYTOGENETICS

Leukemias are associated with changes in chromosome number, translocation and inversions of genetic material. These are associated with activation or increased transcription of oncogenes. Cytogenetics is important in diagnosing, identifying prognostic groups, monitoring progression of disease such as MRD and is integral in WHO classification.

MOLECULAR GENETICS

It is a recent technology that allows analysis of molecular genetics in leukemias. PCR and southern blot techniques are used to study genetic alterations. Genetic profiling of specific leukemia is further expanded by micro array analysis. Molecular genetics is used to identify immunoglobulin in B cells and TCR gene rearrangement and in recent past to study bcr /abl translocation and translocation 15; 17.

Molecular studies are advantageous in that they detect very few populations of malignant cells (as few as 1 – 5 % of cells in a sample) and can also lead to more rapid test completion.

Congenital leukemia: It is strictly defined when diagnosed between birth and 4 weeks of age. AML ranges from 50% to 90% in various studies.

Genomic instability is suggested in a few cases of congenital leukemia that show multiple immunoglobulin heavy chains by recombinant DNA technology. Clinical

manifestations of congenital leukemia differ in varying degrees from older infants and have nodular skin lesion.

CLASSIFICATION

Acute leukemias are phenotypically classified on the basis of retained partial capacity of differentiation, they are basically classified into

- 1) Myeloid (non lymphocytic) and
- 2) Lymphoid

As per the pediatric clinics of North America, morphologic features and cytochemical staining of blast cells are usually sufficient to distinguish ALL from AML but they do not establish the immunologic subtype of cells, a necessary consideration in the comprehensive diagnosis. .

FAB CLASSIFICATION OF AML

The morphologic approach to AML culminated in the development of standard criteria beginning in 1976 by the French/American/British (FAB) working group, in what became known as the FAB classification of AML.

Daniel et al had proposed revised criteria for classification of AML .It is based primarily on morphologic cytochemical and immunophenotypic features of blast cells.

Argyle JC et al quoted the weakness of the FAB classification which includes⁶⁰.

- 1) Interobserver variability,
- 2) The failure to incorporate cytogenetic data into diagnosis, and

3) Poor correlation with clinical outcome

Modification of the FAB classification was made with recognition of new morphologic subsets of AML and it included immunologic confirmation of M0 and M7 subclasses.

M0 - Minimally differentiated AML

Large agranular Blasts that lack definite cytological and cytochemical markers of myeloblasts there is often a stem cell pattern of antigen expression, with CD34 and Tdt positivity.

Incidence is 2 -3% of AML cases⁶¹.

The criteria for Diagnosis of M0 requires less than 3% of MPO-Positive and Sudan black -B+ve cells but more than 20% of leukemia cells expressing myeloid antigens CD13, 33, and 117⁶². It is also found that lymphoid associated antigens CD2, CD7, CD19 may also be expressed in AML M0.

M1 - AML without differentiation

Incidence is 10- 20% cases of AML⁶³. It is more common in adults, with median age of 45-50 years⁶⁴.

Blasts are more than 90% and more than 3% of blasts are peroxidase positive; few blasts show Auer rods and little maturation beyond the myeloblast stage.

M2 - AML with maturation

First described in 1973. Its incidence is 30%. It is commonly seen in children and young adults. Between 5 and 12% of all AML Patients have the t(8;21). Immunophenotypic markers include the presence of myeloid antigens, increased expression of the natural killer cell

associated antigen CD56, and less commonly, the B cell antigen CD19^{65,66}.

t (8;21) may be predicted by typical morphology, including prominent auer rods, auer rods in maturing neutrophils, marrow eosinophilia with salmon colored granules, cytoplasmic globules and vacuoles, abnormal secondary granules that partially obscure the nucleus, giant granules, rim of peripheral cytoplasmic basophilia, and bilobed neutrophils⁶⁷.

M3 - Acute promyelocytic leukemia

Incidence is 5-10%⁶⁸. Most patients are younger, mean age 30 to 38 years.

The morphologic features of M3 blasts are, nuclei of most cells are bilobed or reniform; hyper granular abnormal promyelocytes with bundles of auer rods are present. Confusion with an atypical monocytic leukemia is frequent; M3v variant is characterized by deeply notched nucleus with few fine granules and infrequent Auer rods.

The myeloid antigens CD13 and CD33 are present but HLA DR is absent; the micro granular variant commonly expresses CD34 and the T cell antigen CD2⁶⁹.

M4 - Acute myelomonocytic leukemia

Incidence is 16-25%, with a median age of 40 to 45 years. Myelocytic and monocytic differentiation is evident, 20%-80% of non erythroid cells are monoblastic, myeloid elements show range of maturation, M4EO variant is associated with >5% dysplastic eosinophils in marrow⁷⁰.

Monoblasts are positive for nonspecific esterase. When stained with PAS, the eosinophilic granules themselves are positive, whereas normal eosinophils demonstrate only

inter granular staining.

M5 - Acute monocytic leukemia

Incidence is 10 %. In M5a subtype, monoblasts predominate in marrow and blood; in M5b subtype, mature monocytes predominate in the peripheral blood⁷¹.

Patients with M5a tend to be younger (75% < 25 years of age) than those of M5b, but both include a wide age range.

Ultra structural studies are helpful to differentiate pure monocytic form from the myelomonocytic variety. It shows that monoblasts and more mature monocytoïd forms have an extreme nuclear irregularity, frequent nuclear bridges and blebs, and abundant cytoplasm with many dense granules, pinocytic vesicles and vacuoles.

Elevated levels of lysozyme are found in more than two-third of patients and may contribute to renal insufficiency or proteinuria .

M6 - Acute erythroleukemia

Dysplastic erythroid precursors (some megaloblastoid, others with giant or multiple nuclei) predominate, and within the non erythroid cells >30% are myeloblasts

Erythroleukemia usually occurs in patients of 50 yrs age or older and is more common in men .

The presenting complaints are constitutional, including fatigue, malaise, and other complaints related to anemia, but in approximately one-third of patients, bone pain may be a major symptom.

In some cases of M6, the erythroid cells may show strong cytoplasmic PAS positivity. This is granular in early erythroid precursors and diffuse in later stages. Erythroid precursors

are PAS negative in normal individuals and in most of the diseases, including megaloblastic anaemia. They are sometimes positive, however in iron deficiency anemia, thalassemia, and refractory anaemia with ring sideroblasts.

M7 - Acute megakaryocytic leukemia

AMgL was added to the FAB classification in 1985. It represents 5-10% of cases of AML⁷².

Megakaryoblasts react with platelet specific antibodies directed against GPIIb/IIIa or VWF; Serum lactate dehydrogenase levels are often elevated with osteosclerotic and osteolytic lesions being demonstrated radiographically.

Myelofibrosis or increased marrow reticulin are seen in most cases. Cytopenias are usually present, but approximately 30% of patients have platelet counts that exceed 1,00,000/cu.mm⁷³.

WHO classification recognizes chronic eosinophilic leukemia with the overlapping hypereosinophilic syndrome and acute basophilic leukemia and the spectrum of mast cell disease including mast cell leukemia

WHO CLASSIFICATION OF AML

AML with recurrent cytogenetic abnormalities

It has a flat incidence through out life with median age in 30s .They constitute 85% of patients with AML in young patients and a meagre percent of elderly patients. AML with t (8; 21) (q22; q22) (AML1/ETO), constitute 10% to 15% of cases of AML and exhibits FAB M2 morphology.

AML with inv (16) (p13;q22) or t(16,6) (p13;q22) (CBFb/MYH11), constitutes 6 to 8%

of AML cases. It is associated with FAB M4Eo morphology

Acute promyelocytic leukemia [AML with t(15;17) (q22;12), (PML/RAR-a) and variants. It constitutes 8-10% of AML cases. There is a high correlation with M3h or M3v morphology. 2 to 3% cases have M1 or M2 morphology.

AML with 11q23 (MLL) abnormalities. It is associated with FAB M5 morphology. It represents 5-8% of cases.

AML with multilineage dysplasia

Myelodysplastic syndrome (MDS) type dysplastic features should be present in at least 2 cell lines

AML and myelodysplasia syndrome, therapy-related

Alkylating agent-related

Topoisomerase II inhibitor-related

AML not otherwise categorized

AML minimally differentiated

AML without maturation

Acute myelomonocytic leukemia

Acute monocytic leukemia

Acute erythroid leukemia

Acute megakaryocytic leukemia

Acute basophilic leukemia

Acute panmyelosis with myelofibrosis

Myeloid sarcoma

Acute leukemia of ambiguous lineage

Biphenotypic acute leukemia

Undifferentiated leukemia.

Categories of AML with recurrent cytogenetic abnormalities of t(15;17), t(8;21), inv(16)/t(16;16), and 11q23 showed significant differences in 5-year survival. No significant difference was identified between AMLs arising from myelodysplasia and de novo AML with multilineage dysplasia, but all cases with multilineage dysplasia had a worse survival than all other AML and other AML without favourable cytogenetics. FAB types M0, M3, and M4Eo showed differences in survival compared with all other FAB types, with M0 showing a significant association with high-risk cytogenetics and 11q23 abnormalities. Other FAB groups and WHO AML, not otherwise categorized subgroups did not show survival differences.

Multiparameter analyses, including immunological and ultrastructural assays, were important in arriving at the appropriate diagnosis of acute leukemia of ambiguous lineage in the new WHO classification.

FAB CLASSIFICATION OF ALL

FAB in 1976 classifies acute lymphoblastic leukemia (ALL) into three types based on morphology as ALL L1, L2, and L3 as described before.

FAB classification of ALL has got no prognostic significance.

L1& L2 morphological types bear no relationship to immunological type or other prognostic factors.

Löffler H et al had noted that the identification of the L3 variant is of major importance. . According to studies there is a high but not universal correlation of the L3 phenotype as defined by morphology with the immunologically defined B-ALL with surface

expression of immunoglobulins.¹³

The prognostic significance of being able to distinguish between L1 & L2 morphologic subtypes has never been fully proven. Findings with established prognostic significance, such as favourable and unfavourable cytogenetic alterations occur in both L1 & L2. Similarly immunophenotypes that may be of prognostic significance do not correlate well with L1 & L2 morphology⁷⁴.

It has been proposed in the recent WHO classification scheme that the L1 & L2 groups should be combined and designated ALL of L1 and L2⁷⁵.

In contrast with T-cell ALL, lymphoblastic lymphomas of the precursor T-cell lineage usually have no or a minimal peripheral blood or bone marrow involvement and have normal or minimally decreased levels of hemoglobin, white blood cells, and the platelets⁷⁶.

Arbitrary criteria such as the presence of the more than 25% blasts in the bone marrow also have been used to distinguish ALL from lymphoblastic lymphomas⁷⁷.

Lymphoblast lymphomas and ALL have more similarities than differences in pathology, immunophenotype, and genotypes and hence they have been regarded as process falling within the spectrum of a single disease entity. ALL and lymphoblast lymphomas are grouped under the category labeled lymphoblastic leukemia / lymphoma in the Revised European and American Classification of Lymphoid Neoplasm's (REAL) and in the WHO Classification Scheme

PAS (Periodic Acid Schiff) negativity and focal AP (acid phosphatase) positivity, as well as white blood cell count and serum lactic dehydrogenase levels, were significantly related to T-ALL when compared with B-ALL, on the contrary, no statistically significant difference

was demonstrated in the clinical outcome.

Hoelzer D et al in their analysis concluded that there are some serious pitfalls of the morphology in T-ALL, clearly indicating the need for immunological analysis of the leukaemic cells. However, morphology remains an essential component of the diagnostic repertoire, especially when the marrow is difficult to aspirate and in cases with equivocal immunological findings. Furthermore, recognition of a separate myeloid leukaemic component in addition to the lymphatic one requires a morphological analysis.

Despite the advent of modern ancillary techniques for ALL diagnostic evaluation, morphologic examination and cytochemical staining of well-prepared air-dried bone marrow and peripheral blood smears are critical in the pathologic diagnosis and classification of acute leukemias.

An important aspect for the FAB classification of acute leukemia is the cytochemical reactivity pattern of the blasts, Unlike acute myelogenous leukemia (AML), no single cytochemical test is specific for ALL, ALL is negative for myeloperoxidase in cytochemistry studies and lacks staining for the myeloperoxidase protein. Occasionally the morphologic distinction between L1/L2 and L3 may be obscured because L1/L2 blasts may sometimes show significant cytoplasm vacuoles.

The WHO classification does not group the ALL together, but separates them under three broader categories of lymphoid disease:

1. Precursor B cell
2. Precursor T cell
3. Mature B cell neoplasms

In each case it clumps predominantly leukemic disease with predominantly

lymphomatous disease based on similar biologic and genetic characteristic of disease rather than clinical presentation.

The term biclonal or bilineage leukemia is used to indicate a disorder that has two distinct leukemic cell populations One that usually marks with myeloid markers and the other with lymphoid markers.

PROGNOSTIC FACTORS & RISK ASSESSMENT:

ALL:

Favourable risk factors include female sex, age 1 to 9 years, WBC count less than 5,00 cells per cumm, L1 morphology, and hyperdiploidy.

Adverse factors include : male sex, age less than 1 year and more than 9 years, count more than 50,000 cells per cumm, L3 morphology, CNS disease, mediastinal mass, hypoploidy, Philadelphia chromosome and t(4;11), blast on day 7 or 14 after start of therapy. Advanced age and high WBC count at the time of diagnosis have a significantly adverse prognosis. Unfavourable cytogenetic abnormalities in adult ALL include t(9;22), t (4 ;11) and q21;.⁷⁸ T cell ALL is a favourable prognostic factor.

AML:

Favourable factors include t(8;21,t(15;17),inv16.

Adverse factors: Secondary AML or MDS, monosomy 7, extra-medullary leukemia,WBC count of more than 100,000 cells per cumm⁷⁹.

RELAPSE:

25% of children with ALL relapse after treatment. The main sites of treatment are bonemarrow, CNS, and the testes. Features of relapse are a morphological shift from small L1

blasts to larger pleomorphic L2 to L3 blasts, additional changes in cytogenetics occurs in 10% of cases⁸⁰.

SURVIVAL AND PROGNOSIS:

The prognosis in ALL has improved from less than 5% survival before 1965 to 25%-50% during the 70s to 70% during the 80s and 80% for children in the 1990s. In AML the long term survival has increased from 10 to 40%⁸¹.

MATERIAL & METHODS

The present study was carried out in the Department of Pathology, Madurai Medical College, Madurai between Jan 2005 and Jan 2007.

All patients with acute leukemia who were admitted in the Departments of Pediatrics, Medicine & Medical Oncology at Government Rajaji Hospital, Madurai were included in this study.

The clinical and lab profiles were evaluated and were classified into AML/ALL using

FAB classification. The results of 121 patients are presented in the study.

A thorough history concentrating on specific epidemiological pattern, family history, environmental and previous chemotherapy were taken into account. This was followed by detailed physical examination. On suspicion of leukemia the following investigations were performed. Hb, TC, Ultrasound Abdomen, Peripheral smear, Bone marrow aspiration, and special studies like PAS & MPO for all cases. Immunophenotyping and karyotyping were done in selected cases.

Sample collection & processing:

For all patients smears were prepared and air dried and stained by Leishman stain. The blasts were categorized into myeloblast or lymphoblast depending on morphology. Cytochemical staining were done within 24 hours (Annexure II) and they were classified into AML or ALL depending on the staining pattern. Cases that were PAS and MPO negative were analyzed by flow cytometry using panel of markers.

DATA ANALYSIS:

The information collected regarding all the selected cases were recorded in a Master Chart. Data analysis was done with the help of computer using Epidemiological Information Package (EPI 2002). Using this software, frequencies, percentage, mean, standard deviation, χ^2 and 'p' values were calculated. A 'p' value less than 0.05 is taken to denote significant relationship.

Sensitivity and specificity were calculated using the following formulae.

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \times 100$$

$$\text{Specificity} = \frac{\text{True negative}}{\text{False positive} + \text{True negative}} \times 100$$

$$\text{Accuracy} = \frac{\text{True positive} + \text{True Negative}}{\text{No. of cases}} \times 100$$

OBSERVATION AND RESULTS

In the study period from June 2005 to January 2007, 2921 peripheral smear materials were received from Government Rajaji Hospital Madurai. Of these, acute leukemia accounted for 121 cases. Out of these 121 cases, 76 were ALL, 44 were AML, and 1 was biphenotypic leukemia (others).

The overall incidence of ALL was 62.8%, AML – 36.4% and that of biphenotypic leukemia was 0.80%.

Among ALL, ALL-L2 accounted for 60.5% of cases followed by ALL-L1 which accounted for 39.5% of cases. ALL L3 was not encountered in this study. In children L1 and L2 accounted for 50% cases of ALL. In adults, ALL L2 accounted for 75 % and ALL L1 accounted for 25 % of cases (Table 1).

In AML, 59.1% of cases were of AML M2 and 18.2 % of cases were of AML M4 type. AML M5 accounted for 11.3 % of cases. 9.1 % of cases were AML M1 (Table – 1).

AGE:

In children, 57.4% of leukemia cases occurred between 6-12 years age group. The mean

age was 6.2 years in children. (Table – 2).

In patients above 13 years of age, 44.8% of cases occurred between 13 and 20 years; 25.4% occurred between 21 and 30 years. The mean age group in adults is 27.6 years. (Table – 2)

In children, ALL accounted for 79.6% of the cases and AML accounted for 16.6% (L1 & L2). (Table-1)

In adults, the most common leukemia was AML (52.3%) followed by ALL (47.7%). In ALL the difference in incidence of different types of leukemia in children and adults is statistically significant (p value- 0.0001).

SEX:

Out of the 121 cases studied, 83 cases were male and 38 were female. The male female ratio was 2.1:1 (Table – 3).

PRESENTING SYMPTOMS:

Out of 121 cases, 99 cases presented with fever and 25 cases presented with bleeding. Fatigue was present in 27 cases (Table – 4). Two cases of AML presented with proptosis. (Figure -1). There was no statistically significant difference in the presenting symptoms between AML & ALL (p value 0.42).

CLINICAL FEATURES:

Anaemia was present in 85 cases of acute leukemia. Generalized lymphadenopathy was present in 35 cases, of which 28 cases were ALL and 7 cases were AML. Testicular mass was present in 1 case of ALL. FNAC of the mass revealed a hypercellular smear with sheets of

atypical lymphoblasts in a haemorrhagic background. (Figure – 2).

Table – 5 shows the various clinical features in different types of leukemia.

Gingival hyperplasia was seen in 5 cases of AML and in 1 case of ALL. (Figure – 3)

A 13 year old boy with AML presented with multiple congenital abnormalities such as growth retardation, cardiac defects and absent radius (Figure – 4).

Laboratory Findings

Hemoglobin (Hb)

34% of cases of leukemia had Hb level less than 5 gm/dl. 56% of cases of leukemia had Hb levels between 5-10 gm/dl. 10% of cases had counts more than 10gms/dl (Table – 6).

The mean Hemoglobin was 6.26 and 6.56 for ALL and AML respectively.

There was no statistically significant difference in Hb % between AML and ALL cases.

TOTAL COUNT (TC):

5.7% of cases had a total count of less than 10,000. 57.8% of patients had a TC between 10,001- 20,000.

Mean total count at presentation was around 39,000 for acute leukemia cases. There was a statistically significant difference of mean total count in AML and ALL. AML is associated with higher total count. (p value 0.012.)

Hyperleucocytosis was present in 8.3% of cases (Table – 7). Figure – 5 shows peripheral smear of an ALL patient with clinical features of leucostasis. The total count of the patient was above 1, 00,000/cumm.

PLATELET COUNT:

11.7% of leukemia cases showed platelet count below 25,000 cells/cumm.

A normal platelet count of more than 1, 50,000 cells/cumm was seen in 21.6% of cases

of acute leukemia.

Mean platelet count was around 96,283 for ALL and that for AML was 1, 00,116. This difference was statistically insignificant (Table – 8).

PERIPHERAL SMEAR:

14.2% of patients had a blast count of less than 20%, of which 88% were ALL and 12% were AML. Blast in peripheral smear ranging from 20-89% was seen in 67.5% of cases and a blast count of above 90% was seen in 18.3% of cases.

The average percentage of blast in peripheral smear was 60.44%

The mean blast percentage was 68.28 and 56.14 for AML and ALL respectively.

There was no statistically significant difference in percentage of blasts in AML and ALL. (Table – 9)

BONE MARROW:

64.2% of cases of acute leukemia had more than 90% blast in bone marrow 35.8% of cases had blast count between 20% and 89%. There was no statistically significant difference in percentage of blast between ALL and AML. (Table – 10) Figure – 6 shows the low power view of hypercellular marrow with absent fat cells with suppressed megakaryopoiesis and erythropoiesis. The mean percentage of blast in bone marrow was 80.81 and 84.15 for AML and ALL respectively.

MORPHOLOGY:

Out of the 121 cases studied morphologically, 63 cases resembled lymphoblast and 58 cases resembled myeloblast. The Myeloblast showed increased nuclear cytoplasmic ratio with

fine chromatin, 2 – 5 distinct nucleoli and cytoplasmic granules (Figure – 7). Auer rods were more prominent in AML M2 than in AML M1 (Figure – 8). A case of AML M3 was encountered, it showed abnormal promyelocyte with dense granules & faggots in the cytoplasm (Figure – 9). Monoblastic differentiation was observed in AML M4 and AML M5 types. M4 showed myeloblast of 20-80% (Figure – 10). AML M5 showed > 80% monoblast with centrally placed nucleus, pale cytoplasmic vacuolization and perinuclear clearing (Figure – 11).

Among the blast which were diagnosed morphologically as lymphoblast 73% were ALL and 25.4% turned out to be AML after cytochemical and immunophenotyping techniques (one case was biphenotypic leukemia). Lymphoblast showed eccentric nucleus with cleaved indented to regular nuclear membrane, coarse chromatin and indistinct nucleoli. ALL L1 blast were predominantly small cells with scant cytoplasm and coarse chromatin (Figure – 12). ALL L2 blast were composed of large heterogenous cells with moderate amount of cytoplasm (Figure – 13). Occasional L2 blast showed dense granules in cytoplasm (Figure – 14).

Out of the 58 myeloblasts, 51.7% of cases turned out to be ALL and 48.3% of cases were AML.

The accuracy of diagnosing ALL on morphology alone was 61.2% and that of AML on morphology alone was 39.5%. (Table – 11)

CYTOCHEMISTRY:

MPO:

Out of 121 smears stained with MPO, positivity was present in 39 cases. Myeloblast showed dense granular brownish black cytoplasmic positivity (Figure – 15). Auer rods were prominently seen after MPO staining. Intense cytoplasmic staining was observed in AML M3 (Figure – 16). Negative staining was observed in ALL (Figure – 17).

In cases of AML, 88.6% were MPO positive and 11.4% were MPO negative.

The sensitivity, specificity and accuracy of MPO in classifying AML was 89%, 100% and 95.9% respectively. (Table – 12)

SUDAN BLACK B:

Out of 121 smears stained with SBB, positivity was present in 41 cases. A dense smudgy blackish cytoplasmic staining was seen in AML cases (Figure – 18). Intense staining was seen in AML M3 compared with other sub types (Figure – 19).

In SBB positive cases 92.85 % were AML. 6.25 % of SBB negative cases were AML. The sensitivity, specificity and accuracy of SBB in classifying AML was 89%, 97% and 93.4% respectively. (Table – 13)

PAS:

PAS positivity was present in 76 cases of acute leukemia. PAS positivity is shown by block staining pattern (Figure – 20).

Out of the 76 cases which are positive for PAS, 66 were ALL and 10 cases turned out to be AML after further studies.

The blasts in 45 cases were negative for PAS. True negative were 35 cases and false negative was 10 cases.

In cases of ALL 86.8% showed block positivity, and 13.2% of cases were negative for PAS.

In cases of AML, 77.3% were negative for PAS and 22.7% were block positive for PAS.
(Table -14)

The sensitivity, specificity and accuracy of PAS in our study was 73%, 78% and 83.5% respectively.

The sensitivity, specificity and accuracy of various stains are given in Table - 15.

IMMUNOPHENOTYPING:

Immunophenotyping was done in 15 cases. The cases which were MPO and PAS negative were analyzed immunohistochemically. Out of 15 cytochemically inconclusive cases, 10 were positive for lymphoid markers and turned out to be ALL and 5 were positive for myeloid markers and turned out to be AML.

A 2 years old boy with hepatosplenomegaly had blasts that morphologically resembled monoblasts. MPO and PAS were negative in that case. Morphological diagnosis of AML M5 was made. Immunophenotyping revealed the blasts to be positive for pre B cell markers.

A 10 years old female child had blasts that morphologically resembled lymphoblasts, MPO was negative, immunophenotyping revealed it to be Biphenotypic leukemia.

The most common CD markers positive in ALL cases were CD 10 and CD 34.

CD 33 and CD 14 were positive in AML cases.

CYTOGENETICS:

A 10 year old female child presented with massive splenomegaly, hyperleucocytosis and 35% blasts in peripheral smear. Mature and maturing forms of myeloid series were present in

PS and Bone marrow. A diagnosis of childhood CML was made .Cytogenetic studies in this case was negative for Philadelphia chromosome. No other chromosomal anomalies were present .It was classified as AML not other wise specified as per WHO classification.

DISCUSSION

In the present study the incidence of leukemia in Madurai is 0.45% which is comparable with Indian studies quoting an incidence of 0.34% to 1%⁸.

The most common type of acute leukemia in the present study is ALL (62.8%) followed by AML (36.4%) which is comparable with the observations of Kapoore. G in whose study ALL composed 62.03% of cases and AML 37.9% of cases⁹.

In the present study 56.6% of cases of ALL is seen in children and 80% of cases of AML are seen in adults.

In children the incidence of ALL L1 and ALL L2 is equal. In adults L2 morphology is more common accounting for 75% of ALL cases .This is similar to the study conducted by Loffler H et al¹³ in which L2 was common (68%). The comparative study of morphological

distribution of ALL in children and adults is given in the table 16 and 17 respectively.

In the present study AML M2 is most common, accounting for 59.1% of AML cases followed by M4 which accounts for 18.2% of cases. The incidence of M5 is 11.3%. This is comparable with those quoted by David H in his study on Acute myelogenous leukemia¹⁴ in which the most common leukemia was AML M2 followed by M4 and M5. AML M0 was not encountered in this study.

AGE INCIDENCE:

In the present study the most common type of childhood acute leukemia is ALL (79.6%) followed by AML (16.6%). Table 18 compares the incidence of AML and ALL in children in present study with that of Manipur study and Western literature^{8, 11}.

In the present study incidence of infantile leukemia is 11.1%. ALL constituted 66% and AML 44% of infantile leukemia cases. This is similar to the study conducted by Somjee et al at the Department of Medical Oncology TATA Memorial Hospital Mumbai¹⁶. Western literature quotes AML M5 M4 to be more common¹⁵.

In adults, the most common type of leukemia is AML constituting 52.3% of cases .The AML: ALL ratios is 2.57:1 while other reviews quote a ratio of 4:1¹³.

SEX:

In the present study both types of leukemia is more common in males. The male female ratio is 2.5: 1. This ratio is higher than that quoted by Singh et al (1.5:1) in his study¹³.

ETIOLOGY:

There is no identifiable risk factor for ALL in this study. 7% of cases of AML had a definite etiology. 2 cases were associated with congenital disorder and 1 case was therapy

related AML.

As per literature in patients with Down's syndrome, AML is more common in children less than 3 years of age and in neonatal period it may present as congenital leukemia or as Transient myeloproliferative disorder (TMD). Both cases have similar clinical feature and similar blast percentage and are differentiated only by follow up. TMD cases resolves spontaneously while congenital leukemia has a fatal outcome²¹.

In our study, we encountered a 25 days old child with Down's syndrome with 35% blast in PS and 50% blast in BM. The blasts were of myeloid morphology. Unfortunately we lost follow up of the case.

A 13 years old boy with AML M4 morphology presented with multiple congenital anomalies, dysmorphic features, absent radius and growth retardation. In literature there are studies which relate syndromes such as TAR syndrome with AML¹⁹. These syndromes are associated with increased risk of mutations.

A 40 years female operated for liposarcoma and on chemotherapy with methotrexate for the past 4 years developed therapy related AML. As per literature, leukemia onset after alkylating agent exposure ranged from 1 to 28 years and is most common in 5-9 years range²³. They have worse prognosis.

PRESENTING SYMPTOMS:

The most common complaint in 80% of cases in present study is fever followed by bleeding. This is comparable to that of Singh et al study⁸.

There is no statistically significant difference in presenting symptoms between AML and ALL (p value 0.42).

CLINICAL FEATURES

Generalized lymphadenopathy is present in 28 cases of ALL and only 7 cases of AML. This is comparable with studies conducted by Poplack DG in which he concluded that GLP is more commonly associated with ALL than with AML.³⁰

In the present study 4.9% of cases has gingival hypertrophy of which 83.6% are AML. It is more commonly encountered in AML M4/AML M5. It is due to infiltration of gingival tissue by leukemic blasts. As per literature 3-5% of acute leukemia patients develop gingival hyperplasia of which 88.9% are AML³¹. The various clinical features in different types of leukemia is statistically insignificant (p value 0.32).

LABORATORY FEATURES:

In the present study subleukemic presentation, in which abnormal cells are present in peripheral blood, but the total leukocyte count is not elevated is seen in 7 cases of acute leukemia. Boggs DR in a 10 year study found 53 of 322 cases to have a subleukemic presentation²⁵.

In the present study Hyperleucocytosis was present in 8.3% of cases. Patient's with hyperleucocytosis can present with leucostasis with sludging of leukemic cells in capillaries of lungs and brain.

In the present study a normal platelet count is seen in 21.6 % of cases. Rest of them is associated with thrombocytopenia, with 11.7% of cases having a platelet count below 25,000. Marrow infiltration and chemotherapy are the most common causes of thrombocytopenia in acute leukemia cases²⁵.

In the present study a blast count of less than 20% in PS is seen in 14.2% of cases of acute leukemia of these 88.2% are ALL and only 11.8% are AML. In all these cases bone marrow is required for confirmation and typing of leukemia. This is in concordance with the

observation made by Bennet et al who concluded that first in the sequence of diagnosis of acute leukemia is the distinction from other neoplastic and reactive diseases and confirmation of acute leukemia by morphological and cytochemical studies⁶².

MORPHOLOGY:

In the present study the blasts in 63 cases resembled morphologically lymphoblast with false negativity of 30 and false positivity of 17. The false positivity may be due to monoblasts and myelomonoblasts which have clefted nuclei and somewhat condensed chromatin. False negativity may be due to the presence of granules in lymphoblasts (Figure – 14) and presence of occasional 3-5 nucleoli in ALL L2 blasts (Figure – 13).

58 cases in the present study morphologically resembled myeloblast. True positive are 28 cases. Out of the 28 cases monoblastic differentiation is seen. (Figure – 11).

In the present study 34% of cases of AML have auer rods, which are reddish rod like filaments of aggregated lysozymes which are derived from incorporation of primary azurophilic granules into autophagic vacuoles. These are present in 50% of cases of AML as quoted by Ritter J et al⁷⁹. He considered them to be pathognomonic of AML. Other studies quote an incidence of 15 %⁶³.

The sensitivity, specificity and accuracy of morphology in diagnosing ALL is 61%, 62.2% and 61.2% respectively and the accuracy of morphology in diagnosing AML is 39.5%.

As per the studies conducted by Bennett et al, even experienced morphologists were able to classify only 70% of cases. Correlation with morphology is poor because it is more subjective given to observer variation, and is based on qualitative factors.

MPO:

In the present study the sensitivity, specificity and accuracy of MPO in diagnosing AML is 89%, 100% and 95.9% respectively as compared with the study conducted by Cheng et al in 2005 who quoted a sensitivity of 69% and specificity of 100%⁴⁷. False negativity is commonly associated with AML M5 morphology. These monoblasts are negative for MPO and positive for NSE.

As per the work done by Yang O, a MPO positivity of > 3% confirms myeloid lineage hence in almost all cases distinguishing AML from ALL is relatively easy by staining for MPO. However AML M0 M5 and M7 may be negative for MPO for which immunophenotyping is essential⁸².

Biphenotypic leukemia is MPO negative. As per the studies conducted by Raymond Lay et al in cases of biphenotypic leukemia more than 3% MPO positive blasts can occur⁵⁸.

PAS:

Out of 121 cases studied by PAS stain True positive is 66, false positive is 10, false negative is 10 and true negative is 30.

The sensitivity of PAS is 73 % and the specificity is 78 %. Other studies quote a sensitivity of 52% and a specificity of 81%⁵³. The accuracy of PAS in diagnosing ALL is 83.5% in our study.

As per the work done by Lai et al no single cytochemical test is specific for ALL, however it is an integral component in diagnostic evaluation for ALL. Auer rod negative, MPO negative, SBB negative and PAS positivity in a block positive manner is of utility in diagnosing ALL cases⁵⁸.

Sudan Black B:

Out of 121 cases studied by Sudan Black B stain false positive is 2 and false negative is 5. False positivity is due to ALL L2 blast which shows a weak SBB positivity.

Raymond Lai in his study has confirmed that SBB which stains lipid membrane of MPO granule may also show weak positivity in ALL⁵⁸.

The sensitivity, specificity and accuracy of SBB in diagnosing AML cases are 89%, 97% and 93.4% respectively.

IMMUNOPHENOTYPING:

The lineage of most cases of morphologically and cytochemically poorly differentiated acute leukemia can be accurately characterized by immunophenotyping.

In the present study 13% of cases required immunophenotyping for classification. Among them, 10 are positive for lymphoid markers and turned out to be ALL and 5 are positive for myeloid markers and turned out to be AML.

A 2 years old boy with hepatosplenomegaly with blasts that morphologically resembled monoblasts and MPO and PAS negative, diagnosed as AML M5 showed positivity for pre B cell markers by immunophenotyping.

In classification, surface markers are most useful in distinguishing AML and lymphoid neoplasms. They are also useful in distinguishing AML M0, AML M5 and AML M7 and in defining hybrid and biphenotypic leukemias. As per the extensive work done by Bennett JM et al⁶² in 1991, surface markers in AML are most appropriate for the evaluation of morphologically and cytochemically atypical or undifferentiated leukemias or suspected hybrid leukemias.

Snower D.P et al in his study stated that the absence of blasts being positive for

peroxidase is not a reliable indicator for the lymphatic nature of a leukaemia, even if the PAS reaction is typical for ALL. The morphological diagnosis of ALL needs confirmation by immunophenotyping in each instance⁵³.t

In the present study the most common CD markers positive in ALL cases are CD 10 (common lymphoid antigen), CD 34 (precursor antigen) and CD 19 (B cell marker) suggesting a precursor B cell ALL.

In the present study CD 33 (common myeloid antigen) and CD 14 (Monocytoid antigen) are positive in AML cases which are MPO negative and SBB negative.

In this study, a 10 years old female child with blasts that morphologically resembled lymphoblasts, MPO and PAS are negative, immunophenotyping revealed it to be Biphenotypic leukemia. Both myeloid (MPO) and lymphoid (CD 19, CD10) markers are positive

SUMMARY

The salient features observed in the present study are

- 1) In children the most common acute leukemia is found to be ALL.
- 2) In adults the most common acute leukemia is AML.
- 3) Males predominate in both types of leukemia.
- 4) Subleukemic presentation is found in 5.8% of cases.
- 5) In subclassifying acute leukemia, ALL L2 is more common and AML M2 is found to be more common among AML.
- 6) In the present study morphology alone showed an accuracy of 61.2%.
- 7) The percentage of sensitivity, specificity and accuracy are 89%, 100% and 95.9% respectively for MPO stain in AML cases.
- 8) The sensitivity of 73%, specificity of 78% and accuracy of 83.5% is found for PAS stain in ALL cases.
- 9) The cytochemical stains markedly improved the accuracy of identifying AML cases.
- 10) Only 13% of cases required Immunophenotyping to classify into AML and ALL.

CONCLUSION

The diagnosis of acute leukemia entails a stepwise approach. First in sequence and importance is the distinction of acute leukemia from other neoplastic diseases and reactive disorders. Second is differentiating acute myeloid and acute lymphoblastic leukemia. The third facet is the classification of AML and ALL into categories that define treatment and prognostic groups.

The progressive developments in the diagnosis of leukemias have unfortunately not translated into reality in the developing world. There exist huge discrepancies in the diagnosis of leukemias between developed and developing countries as quoted by Maman Chandey

Morphology remains the means by which acute leukemia is initially detected and is a major aid in distinguishing ALL and AML.

Cytochemical reactions provide additional information with which to distinguish between the two distinct acute leukemia entities

With the addition of cytochemistry to the morphologic assessment, most cases of acute leukemia can be appropriately designated as either AML or ALL. However, there remains a significant minority of cases that cannot be definitely diagnosed by these methods.

In a developing country like India where immunophenotyping are not available for poor patients due to socioeconomic constraints, morphology combined with cytochemistry provides an improvement in identification.

In conclusion cytochemistry substantially improved the classification of leukemias which are very beneficial to the patients of Government Rajaji Hospital, Madurai, regarding the

line of treatment.

Immunophenotyping is essential in cases where morphology and cytochemistry are inconclusive.

ACKNOWLEDGEMENT

I hereby sincerely thank and acknowledge Dean, Madurai Medical College, Madurai for having permitted me to use the material from Government Rajaji Hospital and Madurai Medical College to carry out this dissertation work.

BIBLIOGRAPHY

- 1) Murphy SB: Classification, staging and end results of treatment of childhood Non Hodgkins lymphomas: Semin oncol; 7: 332, 1980.
- 2) Henderson ES, Lister TA, Greaves MF. History of leukemia. In: Henderson ES, Lister TA, Greaves MF, eds. Leukemia. Philadelphia: Sanders;1-7, 2002.
- 3) Virchow RLK. Wiesses Blut. N Notiz Geb Natur u Heilk : 36:151,1845.
- 4) Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood ; 100:2292-2302, 2002.
- 5) Brunning RD, Vardiman J, Matutes E, acute myeloid leukemia. In: Jaffe ES, Harris NL,

Stein H, Vardiman JW, eds. World Health Organization classification of tumors: tumors of haematopoietic and lymphoid tissues. Lyon: IARC Press ;75-107, 2001.

- 6) Sharma JS, Mohindroo S. FAB classification of leukemia: a cytochemical study. Indian J Pathol ; 47(3):336-339, 2004 July.
- 7) Harris NL, Jaffe ES , Diebold J : World Health Classification of neoplastic diseases of the haemotopoeitic and lymphoid tissues. J Clin Oncol ; 17: 3835, 2002.
- 8) Singh B.M Singh R War E, Cartwright RA Leukemias in children; Paper presented at Bangalore Pedicon 2002.
- 9) Kapore G. acute leukemias: reference manual for national training project: Practical Paediatric Oncology ;1:41 – 46, 1998.
- 10) Gurney JG, Davis S, Severson RK, et al. Trends in cancer incidence in the U.S. Cancer ;78:532-541, 1996.
- 11) David H, Ebb MD, and Howard J. Weinstein MD. Diagnosis and treatment of childhood acute myelogenous leukaemia. Paediatric Oncology Volume 44. No 4 ; 847-860, 1997.
- 12) Raja T. Recognition and management of childhood acute lymphoblastic leukemia. Indian Journal of practical Paediatrics ;5:323-325, 1997.
- 13) Loffler H, Kayser W, Schmitzn: Morphological and cytochemical classification of adult acute leukemais; Haematol Blood Transfuse 30: 21, 1987.
- 14) Weinstein MD, David H, Ebb MD, and Howard J. Diagnosis and treatment of acute myelogenous leukaemia. Oncology Volume 44. No 4; 847-860, 1997.
- 15) Ebb DH, Weinstein HJ. Diagnosis and Treatment of Childhood Acute Myelogenous Leukemia. The Pediatric Clinics of North America: Pediatric Oncology; 44 : 847-858, 1997.

- 16) Som G, Sapra R, Shindae. Leukemia in infants. Indian Journal of Paediatrics; 69:225-227, 2002.
- 17) Sole F, Caballin MR, Coll MD, et al. Acute lymphoblastic leukemia with t(4;11) in a patient previously exposed to a carcinogen. Cancer Genet Cytogenet ;49:133-136, 1990.
- 18) Cronkite EP. Chemical leukemogenesis: benzene as a model. Semin Hematol ; 24:2-11, 1987.
- 19) Ellis N, Groden J, Ye TZ, The Bloom's syndrome gene product is homologous to RecQ helicases. Cell ; 83:655-666, 1995.
- 20) Taylor GM, Birch JM. The hereditary basis of human leukemia. In: Henderson ES, Lister TA, Greaves MF, eds. Leukemia. Philadelphia: WB Saunders ; 210-245, 1996.
- 21) Zipersky A, Poon A, Doyle J: leukaemia in Down's syndrome: A review. Paediatric hametol Oncol 9:139,1992.
- 22) Pedersen-Bjergaard J, Philip P, Larsen SC, Therapy-related and de novo acute myeloid leukemia. Cancer Genet Cytogenet ; 55:19-124, 1991.
- 23) Pedersen-Bjergaard J. Chemicals and leukemia. In: Henderson ES, Lister TA, Greaves MF, eds. Leukemia. Philadelphia: Saunders 171-199, 2002.
- 24) Bick RL, Strauss JF, Frenkel EP. Thrombosis and haemorrhage in oncology patients. Hematol Oncol Clin North Am ; 10:875-907, 1996.
- 25) Boggs DR, Wintrobe MM, Cartwright GE. Acute leukemias: analysis of 322 cases and review of the literature. Medicine ; 41:163, 1962.
- 26) Desch JK, Smoller BR. The spectrum of cutaneous disease in leukemias. J Cutan Pathol ; 20:407-410, 1993.
- 27) Scott CS, Stark AN, Limbert HJ, et al. Diagnostic and prognostic factors in acute

- monocytic leukaemia: an analysis of 51 cases. *Br J Haematol* ; 69 : 247-252, 1988.
- 28) Blayor WA, Poplack DG. Prophylaxis and treatment of leukaemia in the central nervous system and other sanctuaries. *Semin Oncol*; 12: 131-148, 1985.
- 29) Thimmarayappa NM. Testicular leukemia. *Pediatric haematology review* ; 1(2): 1-2, 1998.
- 30) Poplack DG, Reaman G. Acute lymphoblastic leukemia in Childhood. *Paediatric Clinics of North America: The Leukemias* ; 35(4): 903-925, 1988.
- 31) Curtis L, Ruth RN, Shore . Gingival Hyperplasia Complicating Acute myelomonocytic Leukemia. *J Can Dent Assoc* ; 66:78-9, 2000.
- 32) Mastrangelo R, Poplack D, Bleyer A. Biological basis for staging, stratification and treatment. *Med Paed Oncol* ;14:191 – 194, 1986.
- 33) Hoelzer D. Diagnosis and treatment of adult acute lymphoblastic leukemia. In: Wiernik PH, Canellos GP, Dutcher JP, Kyle RA, eds. *Neoplastic diseases of the blood*, 3rd ed. New York : Churchill Livingstone ; 295-319, 1996.
- 34) Miller D, Krailo M, Bleyer W: Prognostic implications of blast cell morphology in childhood acute lymphoblastic leukemia : a report from the Childrens Cancer Study Group. *Cancer Treat Rep* 69:1211, 1985.
- 35) Hayhoe FGJ, Quaglino D. *Haematological cytochemistry*. Edinburgh : Churchill Livingstone ; 259 – 308, 1980.
- 36) Polliack A, Leizerowitz R. Cytochemistry and ultrastructure in lymphoma and leukemia: utility in the diagnosis of different leukemias and the recognition of subtypes of lymphoproliferative disorders. *Hematol Oncol* ; 1:101-126, 1983.
- 37) Elghetany MT, MacCallum JM, Davey FR. The use of cytochemical procedures in the

diagnosis and management of acute and chronic myeloid leukemia. Clin Lab Med. ; 10(4): 707-20, 1990 Dec.

- 38) Gassmann W, Löffler H, Thiel. Morphological and cytochemical findings in 150 cases of T-lineage acute lymphoblastic leukaemia in adults. German Multicentre ALL Study Group (GMALL). Br J Haematol. ; 97(2) 372-82, 1997 May.
- 39) Brunning RD, McKenna RW. Tumors of the bone marrow. Atlas of tumor pathology. Fascicle 9, Third series. Armed Forces Institute of Pathology, Washington: ;19-142, 1994.
- 40) Bainton D. Neutrophilic granules. Br J Haematol ;29:17-22, 1975.
- 41) Hayhoe, F.G.J., Quagliano, D., and Doll, R. : The cytology and cytochemistry of Acute leukemias H.M. stationary office, London, 1964.
- 42) Doe K, Gryzbac, M, Schumacher H. A new modified rapid noncarcinogenic myeloperoxidase staining method using 4-chloro-1-naphthol. Lab Med ;19:374-375, 1988.
- 43) Kaplow L. Substitute for benzidine in myeloperoxidase stains. Am J Clin Pathol ; 63:451, 1975.
- 44) Cheson BD, Cassileth PA, Head DR,. report of the National Cancer Institute-sponsored workshop on definitions of diagnosis and response in acute myeloid leukemia. J Clin Oncol ;8:813-819, 1990.
- 45) Glick AD, Panicker K, Flexner JM. Acute leukemia of adults. Am J Clin Pathol ; 73(4):459-70, 1980 Apr.
- 46) Robert W. McKenna. Multifaceted approach to the diagnosis and classification of Acute Leukemias. Clinical chemistry 46:1252-1259, 2000.

- 47) Cheng J, 'Zhongguo Shi, Yan Xue. Sensitivity and specificity analysis of the lineage related antibodies in acute leukemia ; 13(2):329-31, 2005 April.
- 48) Sheehan H, Storey G. An improved method of staining leucocyte granules with Sudan Black B. J Pathol ;59:336-337, 1947.
- 49) Lillie R, Burtner H. Stable sudanophilia of human neutrophil leukocytes in relation to peroxidase and oxidase. J Histochem Cytochem ; 1:8-26, 1953.
- 50) Nagan M, Chien K, Lee S. Sudan Black Positivity in acute lymphoblastic leukaemia. Mod Pathol ; 5: 68 – 70, 1992.
- 51) Wislocki G, Rhein gold J, Dempsy E. the occurrence of the PAS reaction in various normal cells. Blood ; 4:562-568, 1949.
- 52) Lysy, Yamlt: cytochemical, histochemical analysis of bone marrow. In Knowles DM: Neoplastic haematopathology ;1097, 1992.
- 53) Snower DP, Smith Br, Munz UZ. Reevaluation of the PAS in acute leukaemia with immunophenotypic analyses. Arch Pathol Lab Med ; 115(4): 346-50, 1991 April.
- 54) Moloney W. the selective enzymocytochemical demonstration of neutrophil myeloid cells. Klin Wochenschr ; 42 :553, 1964.
- 55) Yam L, Li C, Crosby W. Cytochemical identification of monocytes and granulocytes. Am J Clin Pathol ;55:283-290, 1971.
- 56) Li C, Lam K, Yam L. Esterases in human leukocytes. J Histochem Cytochem ;21:1-12, 1973.
- 57) Janckila A, Li C, Lam K, Yam L. The cytochemistry of tartrate-resistant acid phosphatase. Technical considerations. Am J Clin Pathol ; 70:45-55, 1978.
- 58) Bucheri V, Raymond Lai, Cheryl F. Pathological diagnosis of acute lymphocytic

- leukemias. *Haematology/Oncology Clinics of North America*. ; 6:14:1209-1234, 2000.
- 59) Barowitz MJ, Carrol AJ, Shuster JJ. Use of clinical and laboratory features to define sub groups in B precursors ALL: *Cancer Res* ; 131:257-267, 1993.
- 60) Argyle JC, Benjamin DR, Lampkin B, Hammond D. Acute Health nonlymphocytic leukemias of childhood: inter-observer variability and problems in the use of the FAB classification. *Cancer* ; 63: 295-301, 1989.
- 61) Stasi R, DelPoeta G, Venditti A,. Analysis of treatment failure in patients with minimally differentiated acute myeloid leukemia. *Blood* ; 83:1619-1625, 1994.
- 62) Bennett JM, Catovsky D, Daniel MT,. proposal for the recognition of minimally differentiated acute myeloid leukaemia (AML-MO). *Br J Haematol* ; 78:325-329, 1991.
- 63) Stanley M, McKenna RW, Ellinger G, Brunning RD. Classification of 358 cases of acute leukemia by FAB criteria: analysis of clinical morphologic features. In: Bloomfield CD, ed. *Chronic and acute leukemias in adults*. Boston: Martinus Nijhoff ; 147-174, 1985.
- 64) Brunning RD, McKenna RW. Acute leukemias. In: *Atlas of tumor pathology. Tumors of the bone marrow*. Washington: Armed Forces Institute of Pathology; 19-142, 1994.
- 65) Koeffler HP. Syndromes of acute nonlymphocytic leukemia. *Ann Intern Med*; 107:748-758, 1987.
- 66) Hurwitz CA, Raimondi SC, Head DR. Distinctive immunophenotypic features of t(8;21) (q22;q22) acute myeloblastic leukemia in children. *Blood*; 80:3182-3188, 1992.
- 67) Nucifora G, Rowley JD. AML1 and the 8;21 and 3;21 translocations in acute and chronic myeloid leukemia. *Blood*; 86:1-14, 1995.
- 68) Grignani F, Fagioli M, Alcalay M. Acute promyelocytic leukemia: from genetics to

treatment. Blood; 83:12-25, 1994.

- 69) Foley R, Soamboonsrup P, Benger A. CD34-positive acute promyelocytic leukemia is associated with leukocytosis, microgranular/hypogranular morphology, expression of CD2 and bcr3 isoform. Am J Hematol ; 67::34-41, 2001.
- 70) Holmes R, Keating MJ, Cork A. A unique pattern of central nervous system leukemia in acute myelomonocytic leukemia associated with inv (16)(p13q2). Blood; 65:1071-1078, 1985.
- 71) Tobelem G, Jacquillat C, Chastang C. Acute monoblastic leukemia: a clinical and biologic study of 74 cases. Blood; 55:71-76, 1980.
- 72) Peterson BA, Levine EG. Uncommon subtypes of acute nonlymphocytic leukemia clinical features and management of FAB M5, M6 and M7. Semin Oncol; 14(4):425-434, 1987.
- 73) Dharmasena F, Wickham N, McHugh PJ. Osteolytic tumors in acute megakaryoblastic leukemia. Cancer; 58:2273-2277, 1986.
- 74) Darbyshore P J, Lilleyman JS: Granular acute lymphoblastic leukemia of childhood: A morphologic phenomenon. J Clin Pathol 40: 251, 1987.
- 75) Harris NL, Jaffe ES, Diebold. A revised European – American classification of lymphoid neoplasms: Blood 84:1361,1994.
- 76) Nathwani BN, Diamond LW: Lymphoblastic lymphoma: A clinicopathological study of 95 cases. Cancer ; 48:2347, 1981.
- 77) Bernard , Boumsell L, Reinherz EL: Cell surface characterization of malignant T cells from lymphoblastic lymphoma using monoclonal antibodies: Evidence for phenotypic differences between malignant T cells from patients with acute lymphoblastic leukemia

and lymphoblastic lymphoma. Blood 57: 1105, 1981.

- 78) Pui CH Acute Lymphoblastic Leukemia. The Paediatric Clinics of North America. Paediatric oncology; 44(4);831-840, 1997.
- 79) Ritter J, Creutzig u, Schellong G. Risk groups in acute non-lymphatic. Analysis of the results of the cooperative therapeutic studies AML-BRM 78 and 83. Onkologie ; 9(2): 78-82, 1986.
- 80) Arya LS. Acute SH, Sunny L. Epidemiological features of Childhood Cancers in Greater Mumbai. Indian Paediatrics 2001; 38: 1270-1276.
- 81) Pui CH, Evans WE. Acute lymphoblastic leukemia. New England Journal ; 339: 605-615, 1998.
- 82) Yang O. Huh M.D and Sherif Ibrahim M.D, PHP. Role of flow cytometry in Diagnosis and monitoring of disease: Haematology / Oncology clinics of North America; 14: 6, 1251-1255, 2000.

ANNEXURE – I

PROFORMA

NAME : **AGE / SEX :**

ADDRESS :

O.P.NO. **H. NO**

WARD

PRESENTING SYMPTOMS

Fatigue / Weight loss / Fever / Abnormal Masses / Purpura / Bleeding / Bone and joint Pain / Past history /Family history.

CLINICAL FEATURES:

Anemia / Lymphadenopathy / Hepatomegaly / Splenomegaly / Sternal Tenderness / Mediastinal Mass/ CNS Involvement / Fundic Haemorrhage.

OTHER ORGAN INVOLVEMENT:

LAB FEATURES:

Total Count :

Haemoglobin :

RBC Count :

Platelet Count :

Bleeding Time :

Clotting Time :

PERIPHERAL SMEAR STUDY

RBC : Hypochromic / Normochromic / Macrocytic Others

WBC : Count : Increased / Decreased
DC : Blasts ; Promyelocyte; Myelocyte;
Metamyelocyte; Stab; Neutrophil;
Eosinophil ; Basophil; Lymphocyte ;
Monocyte

PLATELETS : Increased / Decreased / Normal
Others :

BONE MARROW:

Site : Sternum / Iliac Crest / Others

Biopsy / Aspiration

Cellularity: Normocellular / Hypocellular /
Hypercellular

Erythropoiesis: Active / Suppressed

Maturation: Normoblastic / Micronormoblastic / Megaloblastic.

Leukopoiesis: Active / Suppressed

Blasts:

Size:

Nucleus:

Location : Central / Eccentric

Shape : Regular/Cleaved/Indented

Chromatin : Fine / Course

Nucleoli : Distinct / Indistinct

1-2 / 2-5 nucleoli

Nuclear Membrane : Fine / Dense

Cytoplasm : Scanty / Moderate / Plentiful

Basophilic / Acidophilic / Vacuoles

Granules / Auer Rods / Others

Megakaryocytes : Present / Absent

Plasmacells

Abnormal Cells

Myeloid / Erythroid Ratio

Morphological Blast - Myeloblast / Lymphoblast

CYTOCHEMISTRY

Myeloperoxidase : Positive / Negative

PAS : Positive / Negative

Sudan Black B : Positive / Negative

Non Specific Esterase:

Immunopheno Typing :

DIAGNOSIS:

ANNEXURE – II

STAINING TECHNIQUES

MPO staining Procedure:

Washburn technique:

Preparation of solution A – 300mg of benzidine and 300 mg of basic fuchsin is taken in mortar and pestle. A few drops of absolute alcohol is added and made a paste. Then 100ml of absolute alcohol is added. In another mortar and pestle 5 gm of sodium nitroprusside crystal is taken and distilled water is added drop by drop. This is a saturated solution. Mix both solutions and store in amber colored bottle.

Preparation of solution B – hydrogen peroxide.

Staining Methods.

Fresh air dried smears are taken. Solution A is filtered and poured on the smear. Wait for 2 – 3 minutes. Then add solution B drop by drop. After 1 min wash it in running tap for 2 mins, counter stain with Leishman after fixing the stain in methanol for 2 min.

SUDAN BLACK B STAIN

I. REAGENTS

1. Fixative: Formal Formalin Vapour (as used in peroxidase stain) 10mts.
2. Sudan Black B Stock Solution:

Sudan Black B powder - 0.3gm

Dissolve in absolute alcohol (ethanol) 100ml

Filter and store in dark bottle at 4 deg C.

Buffer – Alcoholic phenol Phosphate Buffer

- a) Dissolve crystal phenol 16gm in absolute alcohol 30ml
- b) Dissolve hydrate disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$)-0.3gm in distilled water 100ml.

Add (b) to (a) – store in dark bottle at 4deg C.

3. Working Sudan Black B Stain

Stock stain 48 ml add Buffer 32ml – Filter.

4. 70% Alcohol.

5. Giemsa stain 1:10 dilution (as for MGG stain).

II. METHOD:

1. Fix smears in formal alcohol for 1 minute.
2. Wash in tap water very gently (or smear washes off) and air dry.
3. Immerse smears in Sudan Black B working solution in a coplin jar for 45 mins (Keep closed to prevent evaporation).
4. Wash in tap water – Shake off excess water.
5. Wash in 70% ethanol for 5 mins.
6. Wash with tap water.
7. Stain with Giemsa stain (in coplin Jar) for 20 mins.
8. Immerse in buffer water pH 6.8 for 10 mins.
9. Rinse in tap water
10. Air dry.

PAS staining procedure:

Air dried films are fixed for 1 min at room temperature in formalin ethanol fixative solution and then rinsed for 1 min in running tap water. The slides are then immersed in PAS solution followed by rinsing in several changes of distilled water. This is followed by immersion in Schiff reagent. And then wash in running tap water for 5 min. the slides are then counterstained with haematoxylin solution. The smears are then examined under microscopy for positivity in block pattern.

Karyotyping.

5ml of peripheral blood is taken and the WBC is separated. The WBC suspension is incubated at 37 C for three days in a culture media containing phytohaemagglutinin, which stimulates cell division. Colchine is added which arrests the cell division in metaphase. Addition of hypotonic solution swells up the cell and disperses the chromosome and makes them easy to identify and count. Cells are then spread on a slide, stained with Giemsa and photographed with a high power microscope. The individual chromosome is cut from the photograph and the arranged in a orderly manner and the aberrations are noted.

